Plant Physiol. (1995) 108: 1185–1195

Posttranscriptional and Posttranslational Control of Enolase Expression in the Facultative Crassulacean Acid Metabolism Plant *Mesembryanthemum crystallinum* L.[1]

Nancy R. Forsthoefel, Mary Ann F. Cushman, and John C. Cushman*

Department of Biochemistry and Molecular Biology (N.R.F., J.C.C.) and Department of Agronomy (M.A.F.C.), 350 Noble Research Center, Oklahoma State University, Stillwater, Oklahoma 74078–0454

During the induction of Crassulacean acid metabolism by environmental stresses in the common ice plant (*Mesembryanthemum crystallinum* L.), enzyme activities involved in glycolysis and gluconeogenesis, including enolase (2-phospho-D-glycerate hydrolase), increase significantly. In this study, we describe two nearly identical cDNA clones (*Pgh1*a and *Pgh1*b) encoding enolase from the common ice plant. This cytoplasmically localized enzyme is encoded by a gene family of at least two members. The polypeptides encoded by these cDNAs share a high degree of amino acid sequence identity (86.7–88.3%) with other higher plant enolases. Enolase activity increased more than 4-fold in leaves during salt stress. This increase was accompanied by a dramatic increase in *Pgh*1 transcription rate and the accumulation of enolase transcripts in leaves. *Pgh*1 transcript levels also increased in leaves in response to low temperature, drought, and anaerobic stress conditions and upon treatment of unstressed plants with the plant growth regulators abscisic acid and 6-benzylaminopurine. In roots, enolase transcripts increased in abundance in response to salt, low and high temperature, and anaerobic stresses. Surprisingly, we observed no increase in enolase protein levels, despite the increased levels of mRNA and enzyme activity during salt stress. The stress-induced increase in enolase activity is therefore due to posttranslational regulation of steady-state enzyme pools. Our results demonstrate that the stress-induced shift to Crassulacean acid metabolism in the ice plant involves complex regulatory control mechanisms that operate at the transcriptional, posttranscriptional, and posttranslational levels.

The essential and ubiquitous glycolytic enzyme enolase (EC 4.2.1.11) catalyzes the conversion of 2-phosphoglycerate to PEP. In most higher plants, enolase, like other glycolytic enzymes, is present as multiple isoforms localized to the cytosol and to plastids (Gottlieb, 1982; Shih et al., 1986). Multiple isoforms of the enzyme have been documented in developing oilseeds (Miernyk and Dennis, 1982, 1984, 1987), with the ratio of plastid to cytosolic enolase varying considerably depending on the tissue source. For example, in the developing endosperm of castor beans, the plastid-localized form may contribute up to 30% of the total enolase activity (Miernyk and Dennis, 1992), whereas in other tissues, such as mature leaves of Arabidopsis (Van Der Straeten et al., 1991) and castor bean (Miernyk and Dennis, 1992), the chloroplastic form of the enzyme is undetectable. Southern blot analysis has confirmed that enolase is encoded by multigene families in rice, tomato, and tobacco (Van Der Straeten et al., 1991) but apparently by only a single-copy gene in maize (Lal et al., 1991) and Arabidopsis (Van Der Straeten et al., 1991). To our knowledge, only the cytosolic form of this enzyme has been cloned from the C_4 plant *Zea mays* (Lal et al., 1991) and C_3 plants, including tobacco, Arabidopsis (Van Der Straeten et al., 1991), and castor bean (*Ricinus communis*) (Blakeley et al., 1994).

In addition to its essential role in glycolysis and gluconeogenesis, enolase plays specialized roles during plant development, in nonphotosynthetic tissues, and under conditions of anaerobiosis. Enolase plays an important role in developing seeds of castor bean, in which activity levels of the cytosol- and plastid-localized isozymes peak coordinately with maximal rates of storage lipid accumulation (Miernyk and Dennis, 1992). The increased activity of the plastid-localized isozyme reflects the high demand for pyruvate for de novo fatty acid synthesis in tissues, such as developing seeds that accumulate storage lipids (Miernyk and Dennis, 1987). During fruit ripening, increased glycolytic enzyme activities reflect increased demand for carbon flux through glycolysis (Solomos and Laties, 1974; Stitt et al., 1986). During artificially induced ripening, enolase mRNA levels increased 30-fold and enolase enzyme activity increased 3-fold (Van Der Straeten et al., 1991). Exposure of plants to anaerobic stress causes a shift from an oxidative to a fermentative mode of carbohydrate metabolism, resulting in the increased expression of many enzymes of the glycolytic pathway (Dennis et al., 1987). Whereas the first and the last enzymes in the glycolytic pathway are strongly induced, intermediate enzymes in the pathway such as enolase are induced at low levels (Kelley and Freeling, 1984; Bailey-Serres et al., 1988). Enolase has also been shown to be rapidly induced by hypoxia stress in both anoxia-tolerant and -intolerant *Echinochloa* (barnyard grass) spp. (Fox et al., 1993; Zhang et al., 1994).

[1] This work was supported in part by grant No. 92–100–7761 (Plant Responses to the Environment) to J.C.C. from the U.S. Department of Agriculture/National Research Initiative Competitive Grants Program and the Oklahoma Agricultural Experiment Station.

* Corresponding author; e-mail cushman@okway.okstate.edu; fax 1–405–744–7799.

Abbreviations: 6-BAP, 6-benzylaminopurine; enolase, 2-phospho-D-glycerate hydrolase; PEPC, PEP carboxylase.

In the facultative CAM plant *Mesembryanthemum crystallinum*, the induction of CAM, a long-term adaptive strategy to plant survival and reproduction in arid climates, is induced by water stress in the form of high salinity or drought (Lüttge, 1993). PEPC catalyzes nocturnal CO_2 fixation into C_4 acids, which provide a carbon source for refixation via the Calvin-Benson (C_3) photosynthesis cycle during the day when stomata are closed to limit evaporative water loss. In plants performing CAM, carbon flux through glycolysis and gluconeogenesis is enhanced to meet the increased demand for PEP that is required for nighttime CO_2 fixation (Paul et al., 1993). This enhancement involves the increase in activity of many glycolytic enzymes during the transition from C_3 to CAM (Holtum and Winter, 1982; Winter et al., 1982). In particular, enolase activity increases more than 5-fold after 2 weeks of treatment with 400 mM NaCl (Holtum and Winter, 1982). The majority of enolase activity is localized to the cytoplasm, with a small amount associated with the plastid fraction (Winter et al., 1982).

To investigate the molecular basis of the increase of enolase activity during CAM induction, we have isolated and characterized two nearly identical cDNA clones encoding ice plant enolase. We describe the expression patterns of enolase in response to environmental stress and exposure to plant growth regulators. Enolase mRNA expression is induced by salinity stress, primarily at the level of transcription, as measured by in vitro transcription run-on experiments. Despite significantly enhanced enolase enzyme activity, increased transcription rates, and steady-state transcript accumulation during stress treatment, we detected no mass increase in enolase protein levels by immunoblotting during salinity stress treatment. These results indicate that posttranscriptional regulation and posttranslational activation of this enzyme play crucial roles in the complex response of CAM induction.

MATERIALS AND METHODS

Plant Material

Common ice plant (*Mesembryanthemum crystallinum*) seedlings were grown from seed in vermiculite irrigated with half-strength Hoagland solution in a growth chamber on a 12-h light (23°C)/12-h dark (18°C) cycle. Fluorescent lighting provided a photon flux density of 450 to 500 μE m^{-2} s^{-1}. Ten-day-old seedlings were transferred to 5-L black buckets containing Hoagland solution and grown hydroponically with constant aeration. Six-week-old plants were stressed by the addition of NaCl to a final concentration of 400 mM. Drought treatment was imposed by removing plants from the nutrient solution. Low-temperature stress was imposed by transferring the plants directly to 4°C. High-temperature stress was imposed by transferring plants to 40°C. Anaerobic stress was imposed by bubbling argon through the rooting media. Plant growth regulators, ABA and 6-BAP (Sigma), were dissolved in DMSO and diluted to a final concentration of 10 μM in nutrient solution. Control plants for plant growth regulator experiments were treated with DMSO alone. Plant material was harvested at various times after the start of various stress treatments (as indicated in figure legends), frozen in liquid nitrogen, and stored at −80°C until use.

Construction and Screening of cDNA Libraries

cDNA libraries were prepared from poly(A)$^+$ RNA isolated from leaf and root tissues from plants that had been salt stressed for 30 h and from root tissue from plants that had been salt stressed for 6 or 30 h in λ-Uni-ZAP XR (Stratagene) according to the manufacturer's instructions. Libraries were screened using a full-length cDNA clone for enolase from maize (kindly provided by Dr. P.M. Kelly, University of Nebraska) by three rounds of plaque hybridization (Benton and Davis, 1977). Positive λ-Uni-ZAP XR clones were excised and propagated as plasmids according to the manufacturer's instructions (Stratagene).

DNA Sequencing and Sequence Data Analysis

The isolation and restriction analysis of excised recombinant pBluescript SK− (Stratagene) plasmids were carried out using standard procedures (Sambrook et al., 1989). One clone (designated Enol-19) containing an insert of approximately 1.7 kb was isolated and subjected to further analysis. Single-stranded plasmid DNA was made according to the method of Vieira and Messing (1987). Deletion subclones for sequence analysis were generated using the technique of Dale et al. (1985). Four synthetic primers were also used to complete the sequencing of the mRNA-like strand. To sequence the opposite strand, the full-length insert was excised using *Kpn*I/*Xba*I and recloned into these same sites in Bluescript SK−. An oligomer having the sequence 5′-GAACAAAAGCTGGAGCTCCCCCC-3′ was used to linearize the single-stranded DNA with *Sac*I prior to exonuclease digestion with T4 DNA polymerase. Two other clones (Enol-17 and Enol-22) were sequenced using the same synthetic primers used to sequence the mRNA-like strand of Enol-19. All sequencing reactions were performed using the dideoxy chain termination method, with a modified form of T7 DNA polymerase (Sequenase version 2.0) (Tabor and Richardson, 1987).

DNA sequence data were analyzed using the MacVector/AssemblyLIGN sequence analysis program (Eastman Kodak). Data base searches were conducted using the National Center for Biotechnology Information BLAST E-mail server (Altschul et al., 1990). Multiple sequence alignments were conducted using the Clustal multiple-alignment program (Higgins and Sharp, 1989).

Enzyme Activity Assays

Frozen tissue (0.1 g) was homogenized in cold protein extraction buffer (100 mM Hepes, pH 8.0, 0.5% BSA, 0.5% PVP, 5 mM DTT) in a 1.5-mL microcentrifuge tube with a small pestle. Extracts were clarified by centrifugation (15,000*g*, 10 min, 4°C). Soluble protein samples were taken from the supernatants. Samples of soluble protein from unstressed and salt-stressed plants were assayed for enolase activity at 25°C by following the change in A_{340} of a pyridine nucleotide (β-NADH) in a 3-mL reaction mixture

containing 81 mM triethanolamine, pH 7.4, 0.12 mM β-NADH, 25 mM $MgCl_2$, 100 mM KCl, 1.3 mM ADP, 7 units of pyruvatekinase, 10 units of lactate dehydrogenase, and 100 μL of protein extract. Reactions were initiated by addition of 1.9 mM 2-phosphoglycerate. Assays were conducted in the range in which there was a linear relationship between amount of enzyme added and activity detected. The concentration of soluble protein in each sample was determined in duplicate using the Bradford assay (Bradford, 1976), with BSA as the standard.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated as described by Ostrem et al. (1987). For northern blots, 10 μg of total RNA per lane were denatured and resolved on 1.2% agarose gels containing 2.2 M formaldehyde (Sambrook et al., 1989) prior to capillary transfer onto nylon membranes (Magna, Micron Separations, Inc., Westboro, MA). Membranes were UV cross-linked (254 nm) (Stratalinker, Stratagene). Membranes were prehybridized in hybridization buffer (50% formamide, 5× SSC, 2% blocking reagent [Boehringer Mannheim], 20 mM sodium maleate, 0.1% *N*-laurylsarcosine, 0.2% SDS) for 2 h at 42°C and hybridized overnight at 42°C with a 1.7-kb *Kpn*I/*Xba*I insert of Enol-19 (full-length probe) that was digoxygenin labeled using the random-priming method (Feinberg and Vogelstein, 1983). The 3′-specific probe was labeled during PCR amplification using a forward primer (5′-GCATAGATTTCTCAGT-3′) corresponding to positions 1367 to 1383 (italicized in Fig. 1) and a reverse primer (T7 primer, 5′-AATACGACTCACTATAG-3′), which anneals downstream of the pBluescript SK− polylinker. The cycling parameters were: 1 min at 94°C, 1 min at 50°C, and 30 s at 72°C for 30 cycles. The PCR product was purified on a 1.5% agarose gel and isolated with NA45-DEAE membranes (Schleicher & Schuell). Northern blots were processed according to the manufacturer's specifications (Boehringer-Mannheim). Following hybridization, membranes were washed once for 15 min in 2× SSC, 0.1% SDS and twice for 15 min in 0.1× SSC, 0.1% SDS at 65°C. Membranes were blocked with 2% blocking reagent in maleate buffer (100 mM maleic acid, pH 7.5, 150 mM NaCl) and then exposed to antidigoxygenin conjugated to alkaline phosphatase. Membranes were then washed in maleate buffer and exposed to Lumiphos 530 (Boehringer Mannheim) for chemiluminescent reaction. Membranes were exposed to x-ray film (RX, Fuji, Japan) at room temperature for 15 to 60 min.

Genomic Southern Blot Analysis

Total genomic DNA was isolated according to the method of Steinmüller and Apel (1986). After restriction digestion and separation on 0.9% agarose gels, DNA was blotted onto nylon membranes (Magna, Micron Separations, Inc.), followed by 3 min of cross-linking with UV (254 nm) light (Stratalinker, Stratagene). Membranes were then prehybridized in 5× SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% *N*-laurylsarcosine, 0.2% SDS for 1 h at 65°C. The same digoxygenin-labeled full-length and 3′-specific probes described above were used on the Southern blots, except that the probes were diluted in Southern prehybridization solution before use. Membranes were hybridized overnight at 65°C. Membranes were washed for 15 min in 2× SSC, 0.1% SDS at room temperature and then twice for 15 min in 0.1× SSC, 0.1% SDS at 65°C. Detection of the digoxigenin-labeled probes using the chemiluminescent reaction was accomplished as described above.

In Vitro Transcription Assays

Plasmid DNA (5 μg) was slot blotted onto nitrocellulose using a Minifold II apparatus according to the protocol supplied by the manufacturer (Schleicher & Schuell). Isolation of nuclei was carried out according to method II as recently described by Cushman (1995). In vitro transcription assays were conducted as previously described (Cushman et al., 1989).

Western Blot Analysis

Total protein was extracted from leaf and root tissue as previously described (Ostrem et al., 1987). Frozen tissue was homogenized in 100 mM Tris, pH 8.3, 100 mM NaCl, 2 mM leupeptin, 0.5 μg/mL *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane, 10 mM DTT. Ten percent glycerol was added to the soluble fraction before freezing. Total soluble proteins (100 μg/lane) were denatured in preheated SDS sample buffer (100°C for 3 min), resolved by SDS-PAGE (Laemmli, 1970), and electroblotted to nitrocellulose as previously described (Ostrem et al., 1987). Membranes were initially stained with Ponceau S to verify equivalent loading of protein (Sambrook et al., 1989). Enolase was detected by incubating the membranes with rabbit anti-tomato enolase IgG (Van Der Straeten et al., 1991) for 1 h at room temperature, followed by the addition of horseradish peroxidase-linked goat anti-rabbit IgG conjugate (Boehringer Mannheim) for 1 h at room temperature. The protein-antibody complexes were visualized by staining with a solution containing 3,3′-diaminobenzidine and cobalt. Western blots were performed in triplicate, and the relative amount of detectable enolase in the total soluble protein extracts were quantitated directly using a PDI (Huntington Station, NY) digital scanning densitometer.

RESULTS

Isolation and Nucleotide Sequence Determination of Ice Plant Enolase

cDNA libraries were screened using a heterologous cDNA probe from *Z. mays* (Lal et al., 1991). DNA was isolated from positively hybridizing plaques from root (6 h of stress) and leaf libraries (30 h of stress) and screened by restriction analysis for insert lengths equivalent to the expected size of a full-length transcript (approximately 1.7 kb). One clone (designated Enol-19, *Pgh1a*) isolated from the 30-h stressed leaf library was completely sequenced and found to be full length. This cDNA was 1686 nucleotides long with a poly(A) tail of 58 adenine residues. A single open reading frame of 1332 bp began with an AUG

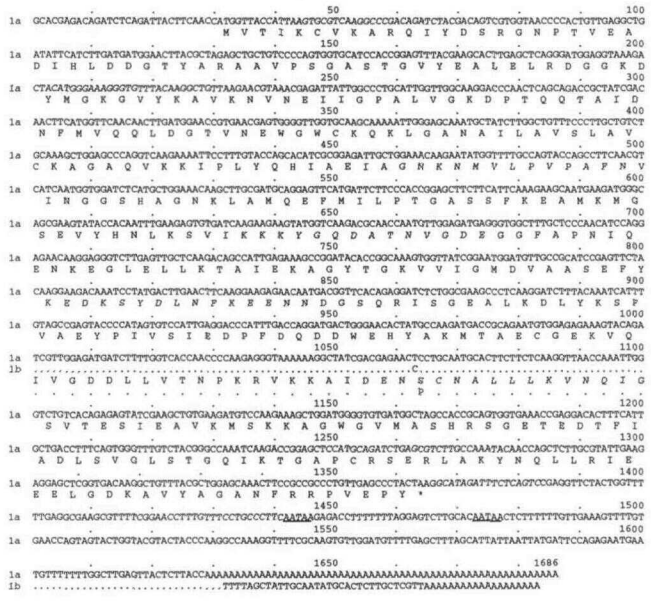

Figure 1. Nucleotide and deduced amino acid sequences of the full-length cDNA for cytosolic enolase from *M. crystallinum*. The deduced single-letter amino acid sequence is given below the center nucleotide of each codon triplet. An asterisk (*) designates the TAG termination codon. Two putative polyadenylation signal consensus sequences are underlined. Italicized sequences designate the oligonucleotide used for generating the 3′-specific probe. The upper sequence is designated *Pgh1*a and the lower sequence is *Pgh1*b.

initiation codon at position 31 and ended with a TAA termination codon at position 1363 as shown in Figure 1. The nucleotide sequence that surrounded the initiation codon at position 31 matched the consensus sequence for plant translational start sites (AACAAUGGC) at seven of nine places (Lütcke et al., 1987). Two polyadenylation signals that closely resembled the consensus polyadenylation signal AATAAA described for higher plants (Wahle and Keller, 1992) were located 76 and 107 nucleotides downstream of the TAA termination codon. Two additional clones, designated Enol-17 and Enol-22 (*Pgh1b*), were approximately 240 bp shorter than Enol-19 and were found to be identical with Enol-19 except for a single base change (T→C) at position 1063 (see Fig. 1), which results in a Ser→Pro substitution. These clones also contained a 36-nucleotide insertion at the 3′ end of the untranslated trailer region as indicated in Figure 1.

Genomic Southern Blot Analysis

To assess the complexity of the enolase gene family in *M. crystallinum*, genomic Southern blots of DNA digested with three different restriction endonucleases were probed either with the *Eco*RI-*Xho*I fragment of the full-length cDNA clone insert (Enol-19) or with a 3′-specific probe generated by PCR, using a primer just downstream of the coding region (designated in italics in Fig. 1). The full-length probe hybridized under conditions of high stringency to at least five fragments in genomic DNA digested with either *Hin*dIII or *Eco*RI (Fig. 2). A single, prominent band was detected in both the *Xba*I and *Eco*RI lanes using a probe specific to the 3′ untranslated coding region. However, two bands were clearly evident in the *Hin*dIII lane.

From these results we conclude that there are two copies of the gene encoding cytosolic enolase present in the common ice plant genome. Our isolation of two distinct cDNA clones for enolase provides further evidence for this conclusion. In most organisms, gene duplication events gave rise to different isozymes of the glycolytic pathway (Gottlieb, 1982; Fothergill-Gilmore, 1986). Therefore, we conclude that the presence of two enolase genes in the ice plant genome is likely the result of a gene duplication event.

Amino Acid Sequence Alignments with Higher Plant and Algal Enolase

The open reading frame shown in Figure 1 encoded a 444-amino acid polypeptide with a predicted molecular weight of 48,284. The deduced protein sequence was aligned with other higher plant and green algal cytosolic enolase sequences as shown in Figure 3. The ice plant enolase was identical in length with *R. communis*, Arabidopsis, and *Lycopersicon esculentum* enolases. The *Z. mays* enzyme was essentially colinear except for a single amino acid (K) addition at position 261 as indicated in Figure 3. The aligned *Chlamydomonas* sequence was colinear for the most part with the higher plant sequences, except that it lacks a 5-amino acid residue stretch (EWGWC, residues

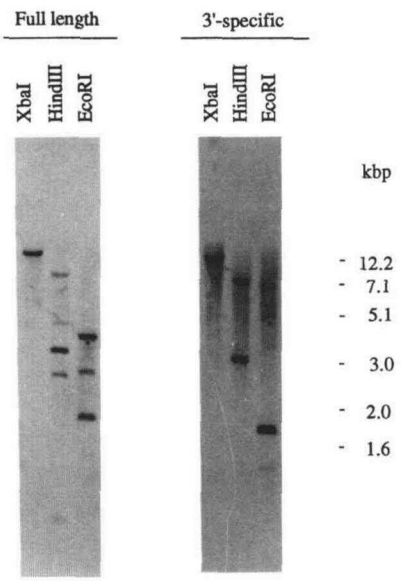

Figure 2. Genomic Southern blot analysis of the enolase gene family in *M. crystallinum*. Total genomic DNA isolated from ice plant leaf tissue was digested with *Eco*RI, *Hin*dIII, and *Xba*I. The DNA samples (5 μg/lanes) were then separated on a 0.9% agarose gel, blotted onto nylon membranes, and hybridized with either a full-length digoxygenin-labeled cDNA probe (pEnol-19) or a 3′-specific PCR-generated probe consisting of a region spanning nucleotides 1367 to the end of the transcript as indicated in Figure 1. Blots were washed under high-stringency conditions (0.1× SSC, 65°C) and then exposed to x-ray film.

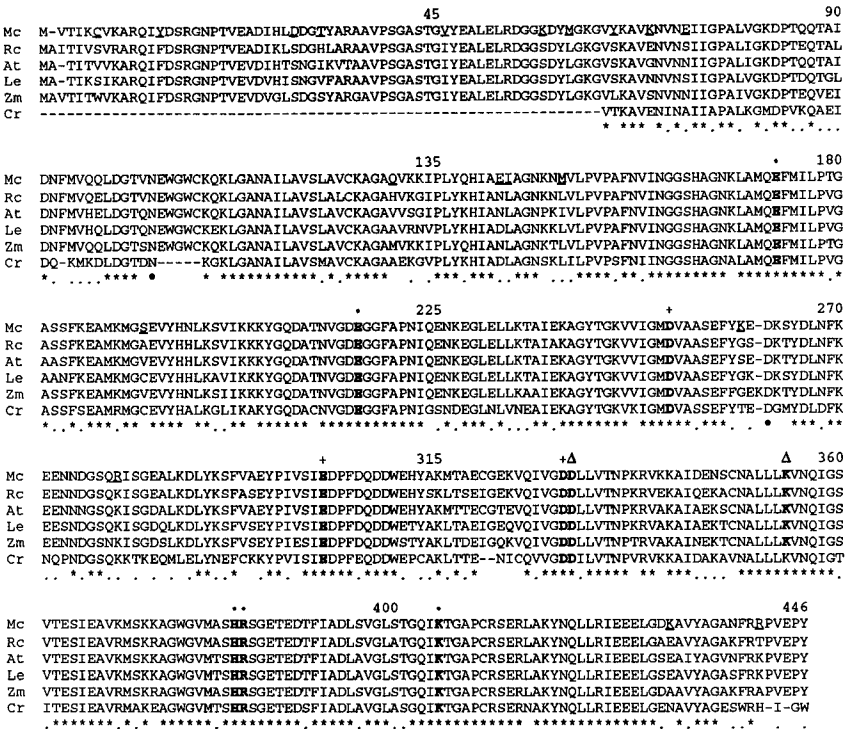

Figure 3. Amino acid alignments of higher plant and green algal enolases. The predicted amino acid sequence of higher plant enolase from *M. crystallinum* (Mc) sequence was aligned to enolase polypeptide sequences from *R.* communis (Rc) (Blakeley et al., 1994), *Arabidopsis thaliana* (At) (Van Der Straeten et al., 1991), *L. esculentum* (Le) (Van Der Straeten et al., 1991), *Z. mays* (Zm) (Lal et al., 1991), and *C. reinhardtii* (Cr) (F. Dumont, unpublished data) using the Clustal multiple-alignment program (Higgins and Sharp, 1989). Sequences are presented in the order of their relatedness to the ice plant sequence. Gaps to optimize alignments are designated by dashes (-). Asterisks (*) indicate consensus amino acid identity among all organisms. Dots (.) indicate positions of conservative amino acid replacement. Residues that are thought to participate in substrate binding (E^{173}, E^{216}, H^{382}, R^{383}, and K^{405}) are indicated with a closed circle (●) (Lebioda and Stec, 1991). Conserved amino acid residues (D^{251}, E^{302}, and D^{329}) that are likely to serve as ligands for conformational Mg^{2+} binding are indicated with plus signs (+). Other amino acid residues (D^{330} and K^{354}) located in the active site of the enzyme (Lebioda et al., 1989) are indicated with triangles (△). Underlined residues are unique to the *M. crystallinum* enzyme.

104–108), 2 amino acids at positions 320 to 321, and single amino acids at positions 93, 442, and 444 (Fig. 3).

In general, enolase, like other enzymes involved in glycolysis and gluconeogenesis, is evolutionarily well conserved. Pairwise comparisons of the predicted primary amino acid sequences from the ice plant and other known higher plant enolases showed 88.3% amino acid identity with castor bean (*R. communis*, Blakeley et al., 1994), 88.0% identity with Arabidopsis (Van Der Straeten et al., 1991), 87.6% identity with maize (Lal et al., 1991), and 86.7% identity with tomato (*L. esculentum*, Van Der Straeten et al., 1991). Alignment of the amino acid sequence data from *Chlamydomonas reinhardtii* enolase (F. Dumont, unpublished data) indicated that it is more distantly related (69.1% identity) to the ice plant sequence.

Enolase Activities Change during Salt-Stress Treatment

To assess the possible changes in enolase enzyme activity during the transition from C_3 to CAM photosynthesis, we assayed enolase levels in soluble root and leaf extracts from salt-stressed *M. crystallinum* plants. In roots, we found that enolase activity levels decreased by more than 30% after 1

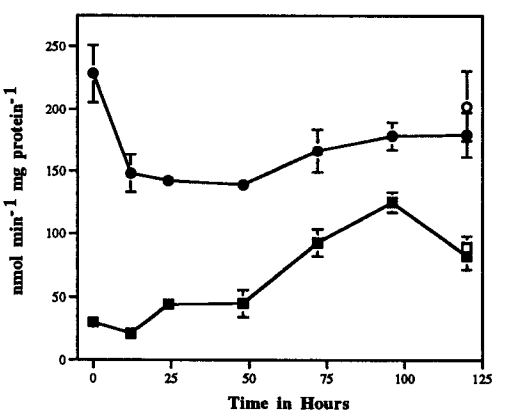

Figure 4. Activity of enolase in leaves and root extracts of ice plant during CAM induction. Hydroponically grown plants were exposed to 400 mM NaCl for the indicated times. The enzyme activities were measured in nondesalted crude extracts from roots (●) and leaves (■) of stressed plants. Activities in roots (○) and leaves (□) of unstressed controls were also determined at the end of the 5-d period. Data are expressed in nmol of β-NADP as measured by changes in A_{340} and represent the average of three (roots) or four (leaves) replicates. Errors bars indicate SE.

to 2 d of stress and then slowly recovered after 5 d of stress (Fig. 4). Control plants showed a reduction (nonsignificant) in enolase levels. Overall activity levels were higher in roots than in leaves, consistent with immunoblotting results (see below). Enolase activity levels in leaves increased more than 80% after 4 d of stress (Fig. 4). Activity in the leaves of unstressed control plants also increased; however, this increase is likely to be due in part to the developmental progression of CAM that occurs as plants grow older (Chu et al., 1990; Cushman et al., 1990).

Enolase Steady-State Transcript Levels Induced by Environmental Stress

To determine the basis for the observed increase in enolase activity, we followed the expression pattern of enolase in the common ice plant by northern analyses of mRNAs isolated from leaves and roots of plants subjected to various environmental stress conditions. Plants grown in hydroponics until 6 weeks of age were treated with either salt, drought, low temperature, high temperature, or anaerobic stress. In all experiments, both a full-length probe (Fig. 5) and a 3′-specific probe (data not shown) hybridized to a single transcript of about 1.7 kb and displayed identical patterns of expression. The 3′-specific probe did not distinguish between the expression profiles of the two different enolase genes, *Pgh1*a and *Pgh1*b.

Under 400 mM NaCl, *Pgh*1 mRNA levels accumulated to 10 times their original levels after 5 d of stress. *Pgh*1 mRNA expression initially decreased after 12 h of salt stress. After this transient decrease, mRNA levels increased rapidly by 24 h of stress and continued to increase throughout the stress period (Fig. 5). *Pgh*1 mRNA levels in leaf tissue from unstressed control plants harvested during the same time course increased just 2 times (data not shown). Enolase mRNAs increased gradually in response to salt stress in roots as well.

Upon exposure of plants to low temperatures (4°C), enolase mRNA levels increased rapidly in leaves and then gradually decline during the 5-d period. In roots, low-temperature stress also resulted in a substantial increase in enolase mRNA expression, although levels decreased on the 5th d of stress.

Drought stress caused a 3-fold increase in steady-state *Pgh*1 mRNA levels in leaves by 6 h, which did not decrease until after 24 h of stress. In roots, *Pgh*1 mRNA levels were already quite abundant and remained fairly constant during drought stress until they declined after 72 h. This decline in root *Pgh*1 mRNA levels was most likely the result of root tissue senescence, since levels of rRNA, as detected by ethidium bromide staining of agarose gels, declined in the latter time points (data not shown).

High-temperature (40°C) stress caused a gradual decline in *Pgh1* mRNA levels in leaves. In roots, however, high-temperature stress caused a significant increase in *Pgh*1 transcript accumulation in roots by 24 h of stress. Anaerobic stress has been shown to induce several enzymes of the glycolytic pathway (Dennis et al., 1987; Bailey-Serres et al., 1988; Zhang et al., 1994). Therefore, the effect of anaerobic treatment in the ice plant was tested and found to increase

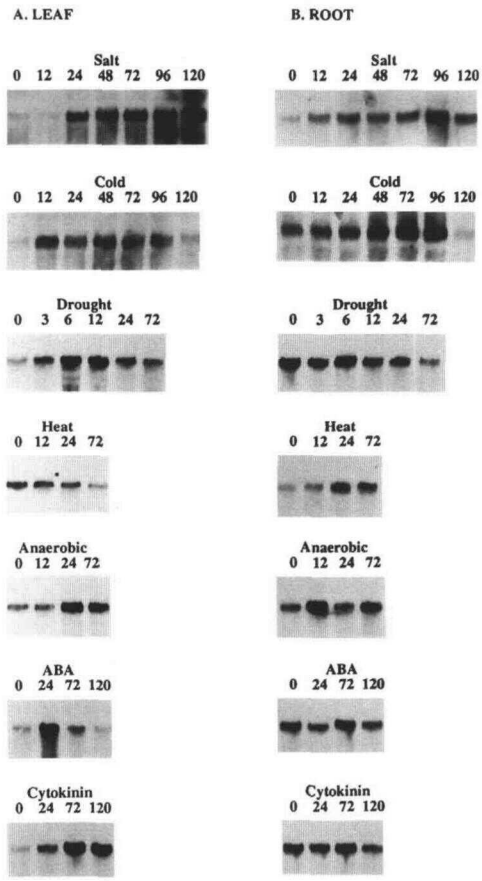

Figure 5. Expression of enolase steady-state transcript levels during environmental stress and plant growth regulator treatments. Hydroponically grown plants were subjected to five different stress treatments: 400 mM NaCl (Salt), plants removed from nutrient solution (Drought), plants exposed to 40°C (Heat), plants exposed to 4°C (Cold), and nutrient solution bubbled with argon (Anaerobic) for the indicated times in h. In addition, unstressed plants were treated with 10 μM ABA (ABA) and 10 μM 6-BAP (Cytokinin). Total RNA was isolated from leaf tissue (A) and root (B) tissue of 6-week-old plants. Total RNA (10 μg) was loaded in each lane and resolved on a 1.2% agarose gel containing formaldehyde. RNA was transferred to nylon membranes and probed with gel-purified, digoxygenin-labeled full-length cDNA insert. The same patterns of expression were observed with both a full-length probe and a 3′-specific probe (data not shown).

enolase expression in leaves after 24 h of stress. In roots, the induction was observed after just 12 h, but levels declined at 24 h and increased again by 72 h.

Recent studies of gene expression in *M. crystallinum* have suggested that expression of certain CAM-related enzymes can be influenced by plant growth regulators (Chu et al., 1990; Thomas et al., 1992; Thomas and Bohnert, 1993; Vernon et al., 1993; Dai et al., 1994). Therefore, the ability of ABA and cytokinin to affect *Pgh*1 expression was tested in unstressed plants. Following exposure of plants to 10 μM ABA in the rooting media, *Pgh1* transcript levels were transiently induced 6-fold within 12 h of application. Roots exhibited a decline in *Pgh1* expression after 24 and 72 h of ABA treatment. Plants showed no obvious signs of stress.

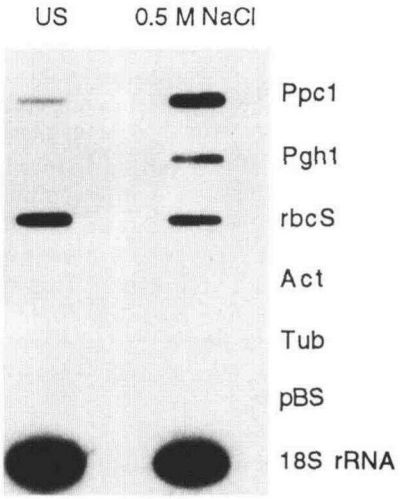

Figure 6. Salt stress increase in *Pgh1* transcription. Nuclei were isolated from either unstressed (US) plants or plants that had been stressed for 120 h with 0.5 M NaCl. Radiolabeled in vitro transcripts synthesized in the presence of [^{32}P]UTP by isolated nuclei were hybridized to 5 μg of cloned (*Pgh1*) (pEnol-19) DNA slot blotted onto nitrocellulose filters. p1B1 (*Ppc1*) was included as a positive control for stress induction, and clones encoding pC3–3 (*rbcS*), actin (Act), tubulin (Tub), pBluescript SK+ (pBS), and 18S (18S rRNA) (pSRI.2B3) from soybean (Eckenrode et al., 1985) were also included as controls.

Addition of 10 μM 6-BAP to the hydroponic solution resulted in a 5-fold increase in the *Pgh1* transcript levels in leaves during a 120-h period. In contrast to ABA-treated plants, plants that received cytokinin treatment appeared wilted and resembled plants that had been salt stressed. The expression of *Pgh1* in root tissue was not affected by 6-BAP.

Transcriptional Activation of Enolase by Salt Stress

To verify that increases in enolase transcript level were due to transcriptional activation of this gene, changes in transcription rates during stress were examined. Amounts of enolase transcripts synthesized by nuclei isolated from leaves of unstressed plants or plants salt stressed for 120 h were quantitated by RNA-DNA slot-blot hybridization. Filters were washed at high stringency (0.1× SSC, 0.1% SDS at 60°C) to distinguish only those transcripts specific for the duplicate enolase genes in ice plant. As shown in Figure 6, salt stress caused a nearly 5-fold increase in transcription rates for enolase in comparison to rates in unstressed leaves. This transcriptional activation was similar to that previously observed for a CAM-specific isogene (*Ppc1*) of PEPC, which was also transcriptionally induced about 6-fold by salt-stress treatment (Cushman et al., 1989). The transcription rates of actin and tubulin (visible upon prolonged exposure of blots, data not shown) remained unchanged during the salt-stress treatment and were used as internal references with which to normalize relative transcription rates. In contrast, we observed a reduction (1.7-fold) in the transcription rate when a gene-specific probe for a member of the *rbcS* gene family from the ice plant was used (Fig. 6).

Immunological Detection of Ice Plant Enolase during Salt Induction

The expression of the enolase genes was examined in both leaf and root tissue during salt-stress treatments. Western blot analyses are presented in Figure 7. Using a rabbit anti-tomato enolase antibody, we detected a 50-kD protein in both leaves and roots of the ice plant. The polyclonal antibody used did not detect any different isoforms of enolase in leaves. In roots, a very faint band is apparent that migrates just above the major immunoreactive species. This faint band may represent an alternate (plastid localized) isoform that is expressed in the roots. Faint bands visible below the major immunoreactive protein in roots are likely to be preoteolytic breakdown products of the enzyme. Enolase is approximately 2- to 3-fold more abundant in *M. crystallinum* roots than in leaves on the basis of total soluble protein content, as measured by activity assays (Fig. 4) and immunoblot analysis (Fig. 7). Quantitation of triplicate immunoblots by digital densitometry (average SE was ± 8.6) indicated that no significant increase in enolase protein levels occurred in leaves or roots during the entire stress period (data not shown).

DISCUSSION

Enolase is encoded by two nearly identical cDNA clones in the facultative CAM plant *M. crystallinum*. In most organisms, glycolytic enzymes have evolved by gene duplication to give rise to different isozymes (Fothergill-Gilmore, 1986). Our isolation and characterization of two nearly identical cDNAs for enolase suggest that a very recent gene duplication event occurred to give rise to two forms of the enzyme. Genomic Southern blots probed with a 3′-specific probe are strongly supportive of the existence of two gene family members (Fig. 2). The presence of a multigene family for enolase in higher plants appears to be widespread. Multiple isozymes for enolase have been described at the protein level (Miernyk and Dennis, 1982) and at the gene level for several different plant species (Van Der Straeten et al., 1991; Blakeley et al., 1994).

Glycolytic enzymes are among the most highly conserved enzymes known and evolve at rates that are slower

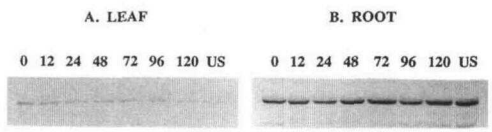

Figure 7. Immunoblot analysis of enolase in leaves and roots of ice plant during salt stress. Total soluble protein was extracted from leaf and root tissue, resolved by SDS-PAGE, and electroblotted to nitrocellulose. Each lane contained 100 μg of soluble protein isolated from leaves (A) or roots (B) of 6-week-old, hydroponically grown ice plants. Plants were stressed for 0, 12, 24, 48, 72, 96, and 120 h with 0.4 M NaCl. Unstressed (US) control plants were harvested after 120 h but received no NaCl treatment. Enolase levels were detected with anti-enolase antibodies from tomato.

than other enzymes (Fothergill-Gilmore, 1986). It has been speculated that CAM plants have evolved from C_3 ancestors multiple times during evolution (Moore, 1982; Monson, 1989). The multiple evolution of CAM suggests that the genetic modifications required for enzymes to function during CAM are likely to be small (Moore, 1982). Furthermore, in facultative CAM plants such as the common ice plant, certain glycolytic enzymes must function during both C_3 and CAM modes of photosynthesis. Consistent with these expectations, we find that the ice plant enolase shows only 19 unique amino acid changes (most located in the N-terminal third of the predicted polypeptide) compared to both C_3 and C_4 versions of enolase (underlined in Fig. 3). Highly conserved amino acid residues that are the ligands for "conformational" Mg^{2+} (Lebioda et al., 1989) that participate in substrate binding (Lebioda and Stec, 1991), that function in enolase catalysis (Brewer et al., 1993), or that participate in the catalytic region of the enzyme in yeast (Lebioda et al., 1989) have been documented (Fig. 3). However, the site at which the two predicted *M. crystallinum* enolases differ (Ser^{347}) does not correspond to any residues known to be crucial for enolase function in yeast.

To investigate mechanisms that control the increase in enolase activity during the switch from C_3 to CAM, we examined the expression of *Pgh1* mRNA under various conditions of environmental stress. CAM can be induced by salinity, drought (Winter and von Willert, 1972; Winter, 1974a), and low-temperature stress, all of which presumably result in reduced water uptake by roots (Winter, 1974b). Indeed, steady-state *Pgh1* transcript accumulation occurs in both leaves and roots in response to high salinity and low temperature and in leaves in response to anaerobic stress (Fig. 5). A transient decrease in *Pgh1* expression occurred in leaves during the initial period of salinity stress (Fig. 5; 12 h). This may have been the result of a salt shock, because the plants wilted and then subsequently regained turgor as adaptive mechanisms to restore water balance were implemented. Drought stress caused a very rapid increase in enolase transcript levels, which gradually declined as the plant tissues began to senesce after 3 d. This very rapid response to drought stress has also been reported for PEPC, which exhibits detectable increases in transcript levels in detached leaves in as few as 2 h (Schmitt, 1990).

Enolase, along with several other glycolytic enzymes, such as phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase, has been reported to have heat-shock-responsive expression patterns in yeast (Iida and Yahara, 1985; Piper et al., 1988). In tomato root and leaf tissues, however, heat shock resulted in a transient decline in enolase mRNA accumulation, followed by a return to normal levels (Van Der Straeten et al., 1991). In the ice plant, enolase mRNA levels declined in leaves upon exposure to heat stress. In roots, however, we detected a significant increase in transcript levels. This pattern of response to heat shock may be involved in the maintenance of glycolytic carbon flux for CAM under the hot, dry conditions of the ice plant's native desert habitat.

In many plant systems, anaerobic stress induces fermentation and a corresponding increase in the expression of many enzymes of the glycolytic pathway. Those enzymes in the middle of the pathway, such as enolase, are induced at low levels (Kelley and Freeling, 1984; Bailey-Serres et al., 1988). In the ice plant, CAM can be induced by anoxia (Winter, 1974b). Accordingly, we observed a clear induction of enolase mRNA levels in leaves and a fluctuating pattern of expression in roots, with increases in transcript abundance at 12 and 72 h after exposure to hypoxic conditions (Fig. 5B). These results are similar to those reported for enolase expression in maize (Lal et al., 1991; Peschke and Sach, 1993), tomato, and Arabidopsis seedlings (Van Der Straeten et al., 1991).

In addition to being responsive to environmental stress, *Pgh1* mRNA expression was transiently induced in leaves by treatment with the plant growth regulator, ABA. In the ice plant, salt stress results in increased levels of endogenous ABA (Thomas et al., 1992). Exogenous ABA treatment reportedly induces CAM characters (Hanscom and Ting, 1978; Ting, 1985), including increased *Ppc1* transcript accumulation (McElwain et al., 1992; Thomas et al., 1992) and increased PEPC and NADP-malic enzyme actvities (Chu et al., 1990; Dai et al., 1994). Although *Pgh1* is clearly regulated by exogenous ABA, we do not know whether this response is solely dependent on increased endogenous levels of ABA. As in the case of *Ppc1* (Thomas et al., 1992) and other water-deficit (Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992) or osmotically responsive genes (Bostock and Quatrano, 1992), we speculate that there are likely to be independent and possibly overlapping signal-transduction pathways from various environmental and developmental events that become integrated to control the ultimate expression levels of enolase.

We initially tested the effect of exogenous cytokinin, because it appears to mimic salt-induced responses in the ice plant such as Pro accumulation, enhanced *Ppc1* gene expression, and the accumulation of pathogenesis-related (osmotin-like) proteins (Thomas et al., 1992; Thomas and Bohnert, 1993). *Pgh1* mRNAs accumulate in leaves in response to cytokinin (6-BAP) treatment. Although cytokinins have been implicated in a wide variety of plant responses, including transcriptional activation of nitrate reductase (Lu et al., 1990, 1992) and PEPC (Suzuki et al., 1994) and increases in PEPC and NADP-malic enzyme accumulation and activity in the ice plant (Dai et al., 1994), their role in controlling gene expression in response to osmotic stress is still not fully understood. One possibility is that cytokinin response elements interact with cytokinin-activated transcription factors (Thomas et al., 1995) to mediate transcriptional activation events.

To confirm that increased steady-state transcripts levels are due to enhanced transcription, we measured in vitro transcription rates for enolase using nuclei isolated from leaves of unstressed and salinity-stressed plants. We found that *Pgh1* transcription rates were 5-fold higher in salt-stressed leaves when compared to leaves of unstressed plants (Fig. 6). The transcriptional activation of *Pgh1* resembles that observed for other CAM-related genes such as

Ppc1 (Cushman et al., 1989), *Mod1* (Cushman, 1992), and *Mdh1* (Cushman, 1993). This observation suggests that transcriptional regulation is the primary mechanism that drives the induction of certain CAM pathway enzymes by salt stress in the common ice plant. However, there is increasing evidence that posttranscriptional events play critical roles in the fine-tuning of gene expression during CAM induction (Cushman et al., 1990; DeRocher and Bohnert, 1993).

Salt-stress treatment results in increased transcription rate, mRNA, and enzyme accumulation of certain enzymes that are important to CAM such as PEPC (Cushman et al., 1989; Michalowski et al., 1989). For enolase, we observed increased transcription rates and mRNA accumulation in response to salt stress; however, we failed to detect any significant changes at the level of protein accumulation. In other plants, enolase mRNA levels are not often correlated with protein levels. For example, enolase mRNA levels in tomato and Arabidopsis roots were estimated to be 10- to 20-fold higher than in leaves, even though enolase protein levels appeared constant in all tissues (Van Der Straeten et al., 1991). In addition, posttranscriptional regulation has been reported for other glycolytic enzymes under conditions of anaerobic stress, such as aldolase (Hake et al., 1985), Suc synthase (McElfresh and Chourey, 1988; Talierio and Chourey, 1989), and wound-inducible genes (Crosby and Vayda, 1991). Modifications in the phosphorylation status of a 31-kD ribosomal protein (Bailey-Serres and Freeling, 1990) and the translational initiation factor eIF-4A (Webster et al., 1991) have been reported and are thought to be associated with translational repression events during anoxia stress. Similar translational repression mechanisms may also play a role in mediating the expression of enolase during CAM induction by salinity stress.

During the induction of CAM by salt stress, certain enzymes such as PEPC, pyruvate Pi dikinase, and NADP-malic enzyme show increases in enzyme activity (Holtum and Winter, 1982) that are accompanied by substantial mass increases in the enzymes themselves (Schmitt et al., 1988). In contrast, despite a significant enhancement in enolase activity in leaves during stress treatment (Fig. 4; Holtum and Winter, 1982), no mass increase in the enzyme was detected immunologically. This suggests that post-translational activation of this enzyme occurs, presumably to permit sufficient carbon flux through glycolysis to support nocturnal C_4 acid production and gluconeogenesis for daytime starch biosynthesis. The in vitro activity of most CAM enzymes measured in unstressed *M. crystallinum* is thought to be sufficient for the increased carbon flux through glycolysis and gluconeogenesis that occurs during CAM (Holtum and Winter, 1982). However, this does not negate the possibility or necessity for higher enzyme activities in vivo for performing CAM.

Roots contained 2 to 3 times more enolase activity and enolase protein than leaves as detected by western blotting. This result is in agreement with the general observation that glycolytic enzyme activities are higher in nonphotosynthetic tissues of mature plants (Goodwin and Mercer, 1983). In Arabidopsis, enolase activities appear higher in roots than in leaves, even though enolase protein levels appeared constant in all tissues (Van Der Straeten et al., 1991). These results suggest that posttranslational mechanisms are operating in roots to maintain high enolase activity. Similar mechanisms also likely occur during climacteric fruit ripening, during which an enhancement in glycolysis does not appear to result from increased enzyme concentrations but rather a retention or activation of preexisting enzymatic activities involved in glycolysis (Solomos and Laties, 1974; Stitt et al., 1986).

At present, the mechanism of enolase activation during CAM induction remains unknown; however, there is evidence to suggest that posttranslational modification involving reversible phosphorylation of the enzyme is involved. Reversible protein phosphorylation is one of the most common mechanisms for modulating enzyme activity. In *M. crystallinum*, phosphorylation of PEPC by a PEPC protein kinase is one of the key mechanisms of modulating diurnal enzyme activity (Baur et al., 1992). In the case of enolase, phosphorylation has been invoked as a possible explanation for the disparity between protein levels and activities found in castor bean (Miernyk and Dennis, 1992). Also, enolase has been shown to be a substrate for phosphorylation by epidermal growth factor-receptor kinase (Reiss et al., 1986) and protein kinase C (Nettelblad and Engström, 1987) in mammalian systems, by a Ser/Thr/Tyr kinase in yeast (Stern et al., 1991) and in *Escherichia coli* (Dannelly et al., 1989). Furthermore, alterations in activity based on changes in phosphorylation state provide compelling evidence for the posttranslational regulation of enolase (Eigenbrodt et al., 1983; Nettelblad and Engström, 1987). Further investigations are needed to elucidate the phosphorylation status of enolase in *M. crystallinum* on a diurnal basis and during CAM induction. The involvement of posttranscriptional and posttranslational modes of regulation in controlling enolase activity in response to environmental stress serves to underscore the importance and complexity of regulatory processes that participate in mediating CAM gene expression.

ACKNOWLEDGMENTS

The authors wish to thank Dr. P.M. Kelly for providing an enolase cDNA clone from *Z. mays* and Dr. D. Van Der Straeten for providing tomato enolase antibodies. The authors would also like to thank Drs. Daniel M. Vernon and Andrew Mort for their helpful comments on the manuscript and the Oklahoma State University Recombinant DNA/Protein Resource Facility for synthesis of synthetic oligonucleotides.

Received November 29, 1994; accepted April 3, 1995.
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The GenBank accession number for the sequence reported in this article is U09194.

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

Bailey-Serres J, Freeling M (1990) Hypoxia stress-induced changes in ribosomes of maize seedling roots. Plant Physiol **94:** 1237–1243

Bailey-Serres J, Kloeckener-Gruissem B, Freeling M (1988) Genetic and molecular approaches to the study of the anaerobic response and tissue specific gene expression in maize. Plant Cell Environ **11:** 351–357

Baur B, Dietz KJ, Winter K (1992) Regulatory protein phosphorylation of phosphoenolpyruvate carboxylase in the facultative crassulacean-acid-metabolism plant *Mesembryathemum crystallinum* L. Eur J Biochem **209:** 95–101

Benton WD, Davis RW (1977) Screening lambda *gt* recombinant clones by hybridization to single plaques *in situ*. Science **196:** 180–182

Blakeley SD, Dekroon C, Cole KP, Kraml M, Dennis DT (1994) Isolation of a full-length cDNA encoding cytosolic enolase from *Ricinus communis*. Plant Physiol **105:** 455–456

Bostock RM, Quatrano RS (1992) Regulation of EM expression in rice. Interaction between osmotic stress and abscisic acid. Plant Physiol **98:** 1356–1363

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brewer JM, Robson RL, Glover CVC, Holland MJ, Lebioda L (1993) Preparation and characterization of the E168Q site-directed mutant of yeast enolase 1. Proteins Struct Funct Genet **17:** 426–434

Chu C, Dai Z, Ku MSB, Edwards GE (1990) Induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* by abscisic acid. Plant Physiol **93:** 1253–1260

Crosby JS, Vayda ME (1991) Stress-induced translational control in potato tubers may be mediated by polysome-associated proteins. Plant Cell **3:** 1013–1023

Cushman JC (1992) Characterization and expression of a NADP-malic enzyme cDNA induced by salt stress from the facultative CAM plant, *Mesembryanthemum crystallinum*. Eur J Biochem **208:** 259–266

Cushman JC (1993) Molecular cloning and expression of chloroplast NADP-malate dehydrogenase during Crassulacean acid metabolism induction by salt stress. Photosynth Res **35:** 15–27

Cushman JC (1995) Isolation of nuclei suitable for *in vitro* transcriptional studies. *In* D Galbraith, D Bourque, H Bohnert, eds, Methods in Cell Biology: Plant Cell Biology. Academic Press, San Diego, CA (in press)

Cushman JC, Meyer G, Michalowski CB, Schmitt JM, Bohnert HJ (1989) Salt stress leads to the differential expression of two isogenes of phospho*enol*pyruvate carboxylase during Crassulacean acid metabolism induction in the common ice plant. Plant Cell **1:** 715–725

Cushman JC, Michalowski CB, Bohnert HJ (1990) Developmental control of Crassulacean acid metabolism inducibility by salt stress in the common ice plant. Plant Physiol **94:** 1137–1142

Dai Z, Ku MSB, Zhang Z, Edwards GE (1994) Effects of growth regulators on the induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* L. Planta **192:** 287–294

Dale RMK, McClure BA, Houchins JP (1985) A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to sequencing the corn mitochondrial 18S rDNA. Plasmid **13:** 31–40

Dannelly HK, Duclos B, Cozzone AJ, Reeves HC (1989) Phosphorylation of *Escherichia coli* enolase. Biochemie **71:** 1095–1100

Dennis ES, Walker JC, Llewellyn DJ, Ellis JG, Singe K, Tokuhisa JG, Wolstenholme DR, Peacock WJ (1987) The response to anaerobic stress: transcriptional regulation of genes for anaerobically induced proteins. *In* D von Wettstein, NH Chua, eds, Plant Molecular Biology. Plenum, New York, pp 407–417

DeRocher EJ, Bohnert HJ (1993) Development and environmental stress employ different mechanisms in the expression of a plant gene family. Plant Cell **5:** 1611–1625

Eckenrode VK, Arnold J, Meagher RB (1985) Comparison of the nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs. J Mol Evol **21:** 259–269

Eigenbrodt E, Fister P, Rubsamen H, Friis RR (1983) Influence of transformation by Rous sarcoma virus on the amount, phosphorylation and enzyme kinetic properties of enolase. EMBO J **2:** 1565–1570

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Fothergill-Gilmore LA (1986) The evolution of the glycolytic pathway. Trends Biochem Sci **11:** 47–51

Fox TC, Andrews DL, Mujer CV, Rumpho ME, Cobb BG, Kennedy RA, Drew MC (1993) Effect of oxygen level on expression of enolase in flooding tolerant and intolerant plants (abstract No. 495). Plant Physiol **102:** S-89

Goodwin TW, Mercer EI (1983) Introduction to Plant Biochemistry, Ed 2. Pergamon Press, Oxford, UK

Gottlieb LD (1982) Conservation and duplication of isozymes in plants. Science **216:** 373–380

Guerrero FD, Jones JT, Mullet JE (1990) Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol **15:** 11–16

Hake S, Kelley PM, Taylor WC, Freeling M (1985) Coordinate induction of alcohol dehydrogenase 1, aldolase, and other anaerobic RNAs in maize. J Biol Chem **260:** 5050–5054

Hanscom Z, Ting IP (1978) Responses of succulents to plant water stress. Plant Physiol **61:** 327–330

Higgins DG, Sharp PM (1989) Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl Biosci Commun **5:** 151–153

Holtum JAM, Winter K (1982) Activity of enzymes of carbon metabolism during the induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum* L. Planta **155:** 8–16

Iida H, Yahara I (1985) Yeast heat-shock protein of Mr 48,000 is an isoprotein of enolase. Nature **315:** 688–690

Kelley PM, Freeling M (1984) Anaerobic capacities of maize fructose-1,6-diphosphate aldolase. J Biol Chem **259:** 14180–14183

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lal SK, Johnson S, Conway T, Kelley PM (1991) Characterization of a maize cDNA that complements an enolase-deficient mutant of *Escherichia coli*. Plant Mol Biol **16:** 787–795

Lebioda L, Stec B (1991) Mechanism of enolase: the crystal structure of enolase-magnesium-2-phosphoglycerate-phosphoenolpyruvate complex at 2.2-Å resolution. Biochemistry **30:** 2817–2822

Lebioda L, Stec B, Brewer JM (1989) The structure of yeast enolase at 2.25-Å resolution. J Biol Chem **264:** 3685–3693

Lu J-I, Ertl JR, Chen C-M (1990) Cytokinin enhancement of the light induction of nitrate reductase transcript levels in etiolated barley leaves. Plant Mol Biol **14:** 585–594

Lu J-I, Ertl JR, Chen C-M (1992) Transcriptional regulation of nitrate reductase mRNA levels by cytokinin-abscisic acid interactions in etiolated barley leaves. Plant Physiol **98:** 1255–1260

Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG initiation codons differs in plants and animals. EMBO J **6:** 43–48

Lüttge U (1993) The role of crassulacean acid metabolism (CAM) in the adaptation of plant to salinity. New Phytol **125:** 59–71

McElfresh KC, Chourey PS (1988) Anaerobiosis induces transcription but not translation of sucrose synthase in maize. Plant Physiol **87:** 542–546

McElwain EF, Bohnert HJ, Thomas JC (1992) Light mediates the induction of phospho*enol*pyruvate carboxylase by NaCl and abscisic acid in *Mesembryanthemum crystallinum*. Plant Physiol **99:** 1261–1264

Michalowski CB, Olson SW, Piepenbrock M, Schmitt JM, Bohnert HJ (1989) Time course of mRNA induction elicited by salt stress in the common ice plant (*Mesembryanthemum crystallinum*). Plant Physiol **89:** 811–816

Miernyk JA, Dennis DT (1982) Isozymes of the glycolytic enzymes in endosperm from developing castor oil seeds. Plant Physiol **69:** 825–828

Miernyk JA, Dennis DT (1984) Enolase isozymes from *Ricinus communis*. Partial purification and characterization of the isozymes. Arch Biochem Biophys **233:** 643–651

Miernyk JA, Dennis DT (1987) Enolase isozymes from developing oilseeds: a survey. J Plant Physiol **128:** 351–359

Miernyk JA, Dennis DT (1992) A developmental analysis of the enolase isozymes from *Ricinus communis*. Plant Physiol **99:** 748–750

Monson RK (1989) On the evolutionary pathways resulting in C4 photosynthesis and crassulacean acid metabolism (CAM). Advances in Ecological Research, Vol 19. Academic Press, San Diego, CA, pp 57–92

Moore PD (1982) Evolution of photosynthetic pathways in flowering plants. Nature **295:** 647–648

Nettelblad FA, Engström L (1987) The kinetic effects of in vitro phosphorylation of rabbit muscle enolase by protein kinase C. FEBS Lett **214:** 249–252

Ostrem JA, Olson SW, Schmitt JM, Bohnert HJ (1987) Salt stress increases the level of translatable mRNA for phospho*enol*pyruvate carboxylase in *Mesembryanthemum crystallinum*. Plant Physiol **84:** 1270–1275

Paul MJ, Loos K, Stitt M, Ziegler P (1993) Starch-degrading enzymes during the induction of CAM in *Mesembryanthemum crystallinum*. Plant Cell Environ **16:** 531–538

Peschke VM, Sach MM (1993) Multiple pyruvate decarboxylase gene in maize are induced by hypoxia. Mol Gen Genet **240:** 206–212

Piper PW, Curran B, Davies MW, Hirst K, Lockheart A, Seward K (1988) Catabolite control of the elevation of PGK mRNA levels by heat shock in *Saccharomyces cerevisiae*. Mol Microbiol **2:** 353–361

Reiss N, Kanety H, Schlessinger J (1986) Five enzymes of the glycolytic pathway serve as substrates for purified epidermal-growth-factor-receptor kinase. Biochem J **239:** 691–697

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schmitt JM (1990) Rapid concentration changes of phosphoenolpyruvate carboxylase mRNA in detached leaves of *Mesembryanthemum crystallinum* L. in response to wilting and rehydration. Plant Cell Environ **13:** 159–171

Schmitt JM, Höfner R, Abou-Mandour AA, Vazquez-Moreno L, Bohnert HJ (1988) CAM induction in *Mesembryanthemum crystallinum*: protein expression. *In* GS Singhal, ed, Photosynthesis—Molecular Biology and Bioenergetics. Springer India and Naroda Publishing, New Dehli, India, pp 259–268

Shih MC, Lazar G, Goodman HM (1986) Evidence in favor of the symbiotic origin of chloroplasts: primary structure and evolution of tobacco glyceraldehyde-3-phosphate dehydrogenase. Cell **47:** 73–80

Solomos T, Laties GG (1974) Similarites between the actions of ethylene and cyanide in initiating the climacteric and ripening of avocados. Plant Physiol **54:** 506–511

Steinmüller K, Apel K (1986) A simple and efficient procedure for isolating chromatin which is suitable for studies of DNase I-sensitive and hypersensitive sites. Plant Mol Biol **7:** 87–94

Stern DF, Zheng P, Beidler DR, Zerillo C (1991) Spk1, a new kinase from *Saccharomyces cerevisiae*, phosphorylates proteins on serine, threonine, and tyrosine. Mol Cell Biol **11:** 987-1001

Stitt M, Cseke C, Buchanan B (1986) Ethylene-induced increase in fructose-2,6-bisphosphate in plant storage tissues. Plant Physiol **80:** 246–248

Suzuki I, Crétin C, Omata T, Sugiyama T (1994) Transcriptional and posttranscriptional regulation of nitrogen-responding expression of phospho*enol*pyruvate carboxylase gene in maize. Plant Physiol **105:** 1223–1229

Tabor S, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci USA **84:** 4767–4771

Talierio EW, Chourey PS (1989) Post-transcriptional control of sucrose synthase expression in anaerobic seedlings of maize. Plant Physiol **90:** 1359–1364

Thomas JC, Bohnert HJ (1993) Salt stress perception and plant growth regulators in the halophyte *Mesembryanthemum crystallinum*. Plant Physiol **103:** 1299–1304

Thomas JC, McElwain EF, Bohnert HJ (1992) Convergent induction of osmotic stress-responses. Abscisic acid, cytokinin, and the effect of NaCl. Plant Physiol **100:** 416–423

Thomas JC, Smigocki AC, Bohnert HJ (1995) Light induced expression of *ipt* from *Agrobacterium tumefaciens* results in cytokinin accumulation and osmotic stress symptoms in transgenic tobacco. Plant Mol Biol **27:** 225–235

Ting IP (1985) Crassulacean acid metabolism. Annu Rev Plant Physiol **36:** 595–622

Van Der Straeten D, Rodrigues RA, Goodman HM, Van Montagu M (1991) Plant enolase: gene structure, expression, and evolution. Plant Cell **3:** 719–735

Vernon DM, Ostrem JA, Bohnert HJ (1993) Stress perception and response in a facultative halophyte; the regulation of salinity induced genes in *Mesembryanthemum crystallinum*. Plant Cell Environ **16:** 437–444

Vieira J, Messing J (1987) Production of single-stranded plasmid DNA. Methods Enzymol **153:** 3–12

Wahle E, Keller W (1992) The biochemistry of 3′-end cleavage and polyadenylation of messenger RNA precursors. Annu Rev Biochem **61:** 419–440

Webster C, Gaut RL, Browning KS, Ravel JM, Roberts JKM (1991) Hypoxia enhances phosphorylation of eukaryotic initiation factor 4A in maize root tips. J Biol Chem **266:** 23341–23346

Winter K (1974a) Einfluss von Wasserstress auf die Aktivität der Phosphoenolpyruvat-Carboxylase bei *Mesembryanthemum crystallinum*. Planta **121:** 147–153

Winter K (1974b) NaCl-induzierter Crassulaceen-Säurestoffwechsel bei der Salzpflanze *Mesembryanthemum crystallinum*. Abhängigkeit des CO_2-Gas-wechsels von der tag/nacht temperature und von der Wasserversorgung der Pflanzen. Oecologia **15:** 383–392

Winter K, Foster JG, Edwards GE, Holtum JAM (1982) Intracellular localization of enzymes of carbon metabolism in *Mesembryanthemum crystallinum* exhibiting C3 photosynthetic characteristics or performing Crassulacean acid metabolism. Plant Physiol **69:** 300–307

Winter K, von Willert DJ (1972) NaCl-induzierter Crassulaceensäurestoffwechsel bei *Mesembryanthemum crystallinum*. Z Pflanzenphysiol **267:** 166–170

Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol **33:** 217–224

Zhang F, Lin J-J, Fox TC, Mujer CV, Rumpho ME, Kennedy RA (1994) Effect of aerobic priming on the response of *Echinochloa crus-pavonis* to anaerobic stress. Plant Physiol **105:** 1149–1157